Original Article

A Modified ATP Assay for Test the Potency of BCG Vaccine

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Abstract

Existing methods for BCG vaccine approval need to be improved to increase accuracy. We focus on the conduction of a more efficient ATP assay procedure to a more accurate quantity of viability of the BCG vaccine by the use of hot Tris-EDTA buffer and DMSO for ATP extraction and also utilization of EnSure device as a more sensitive bio luminometer. The aim was to increase the amount of released ATP during extraction; limit of ATP detection and quantification. The better results in comparison to current ATP assays indicated that this approach was more sensitive and applicable for the determination of the potency of the BCG vaccine and it is suitable for counting of BCG bacilli in low viability samples.

Keywords: ATP, BCG Vaccine, Tris-EDTA

Introduction

BCG vaccine based on one of the attenuated strains of *Mycobacterium Bovis* (BCG) is widely used for vaccination against tuberculosis, immunotherapy of superficial bladder cancer, and perhaps for other immune-therapeutic purposes i.e. against prostate cancer [1-4]. Since the use of the BCG vaccine will continue, either as a stand-alone or as a prime vaccine in prime-boost immunization strategy, the World Health Organization (WHO) has underlined the necessity for further work toward better characterization, evaluation and quality control of which [5]. The potency which is the major characteristic of the BCG vaccine is measured indirectly by bioassay methods, O_2-uptake measurement by the use of the Warburg apparatus, tetrazolium salt assay, ATP assay, and the conventional colony-forming unit (CFU) determination [4]. Believe in being the CFU assay as a time consuming and low accurate due to the tendency of BCG bacilli to aggregation, has compelled the manufacturers to look for a suitable alternative method [4]. In this regard and taking into account the researches over the past five decades ATP assay as a simple, effortless, rapid, sensitive and reliable is the alternative [6, 7] but despite WHO's approval, CFU counting is that remained as the main viability test method which was widely accepted as surrogate for the potency test of BCG vaccine.

Although variability and poor accuracy are cited as the weakness of the CFU method, routinely, the ATP assay also is being correlated and validated by the use of the same aggregated BCG samples based on their CFU which might be supposedly controversial. The tendency to clumping of BCG cells is the most reason for less repeatability of the results whether in CFU or ATP assay [8]. Overall, ATP assay seems to associate with its variability due to being highly dependent on strain, sample state, technical, reagent, nature and size of the cells [6, 8].

The limit of detection (LOD ≈ 30pg ATP equal to 10^5 CFU) and limit of quantitation (LOQ ≈ 1-8×10^3 CFU) in the current ATP assays are about lower than those in the CFU method. The low volume of the prepared sample and the low amount of released ATP are two restrictive factors [7].

In the present study, we aimed to improve the sensitivity and accuracy of ATP assay for BCG count by combined reagents

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for better ATP extraction and using the EnSure (USA) ATP luminometer to test more volume of the samples.

Material and Method

Preparation of BCG Bulk and Vaccine

According to the routine procedure [4], preparation of BCG bulk in 1.5% of monosodium L-glutamate (40 mg/ml) was initiated with culturing of BCG sub-strain Pasteur 1173P2 on Sauton broth. Fresh BCG bulk was diluted (4mg/ml) and dispensed (0.5ml) in 2r amber vials. Partially stoppered vials loaded in freeze-drier. The vials were sealed at the end of the cycle. Samples of vaccines were kept at 2-8˚C until the test.

Determination of CFU

BCG vaccines were reconstituted with 2ml of diluted Sauton media and incubated at 37ºC for 12hrs. the samples then diluted more with normal saline containing 0.5% tween80 to dispense BCG bacilli and to improve the CFU detection to make an actual correlation with ATP. The suspensions were inoculated onto Löwenstein Jensen medium then CFU/mg was counted following incubation at 37 °C for 21-28 days [4].

Calibration of EnSure ATP Luminometer System

An ATP calibration curve was constructed to demonstrate the sensitivity and limit of detection of the systems and the ratio of RLUs to ATP concentration. A 5 pmol ATP standard solution was prepared by diluting the bulk solution with sterilized deionized (DI) water and stored at -20°C. The working volumes for the standard curve were 0, 5, 10, 25, 50, 100 and 200 µL, adjusted to a final reaction volume of 500 µL with DI water. The corresponding amounts of ATP for creating the standard curve were 0, 75, 125, 250, 500, 1000 and 2000 fmol with relative luminescence units (RLU) reading. Ten repeat measurements were taken at each ATP standard. The sample size was 100 µl into the test tube.

ATP Extraction

A 200µl of reconstituted BCG vaccine containing 0.5 unit apyrase was transferred to a 2ml Eppendorf tube containing 100 µL of Dimethyl sulfoxide (DMSO) The tube was shaken by vortex for a second then 400 µL pre hot (99˚C) Tris-EDTA was added to the mixture and tube shook for 6 minutes in boiling water bath. The ATP measurement was done following keeping the tube at room temperature for 1 minute [7].

ATP Measurement

According to the manual sheet, The luminometer was turned on to be activated, calibrated and ready to use. The ATP testing device (MicroSnap total) was removed from the test tube. A 100µl of BCG lysate was added to the test tube then the device was placed back. To enable, immediately or after a few seconds, the plastic valve at the top of the device was broken by bending the bulb backward and forward. The bulb was squeezed twice to expel the liquid (400µl) in the bulb to the bottom of the tube. This liquid helped to release some more ATP from the sample. The entire test device was placed into the bio luminometer and the lid was closed. The result was appeared as RLU on the screen in 15 seconds after pressing 'OK'. Measured ATP in untreated BCG bulk as the free ATP was subtracted from obtained ATP value. For Calibration of Measurement of the bioluminescence signal, the positive and negative control test tubes were supplied with the EnSure company to calibrate the instrument.

Sensitivity Test

Sensitivity is defined as the Limit of ATP Detection (LOD) above the background noise in the absence of ATP. ATP at different concentrations (<100 fmol) was pipetted onto the test tube and read until the point in which the system no longer detected the ATP as a changing in RLU signal higher than any possible background noise.

Reproducibility

This is also referred to as the coefficient of variation (CV). It was calculated using data from 10 replicates for one extraction of the BCG sample in each of the three methods of extraction.

Statistical Analysis

All measurements were performed in minimum triplicate. Mean (X), standard deviation (SD), Coefficient of variation (CV) and p-value for the bioluminescent signal (RLU) and the relative BCG/mg were calculated using Excel software (Microsoft® Office Excel 2016) and Minitab 17. The ATP content was calculated and expressed as RLU per 1 mg of BCG cells.

Results

Preparation of Standard Curve

In establishing the standard curve (Fig.1) by the use of the EnSure luminometer, a strictly linear ratio of RLUs to ATP concentration over the range of 0 to 2000 fmol was obtained. This was tested by constructing an ATP calibration curve in which the sensitivity and the LOD of the system also were determined as equal to 1 fmol ($p \leq 0.01$).

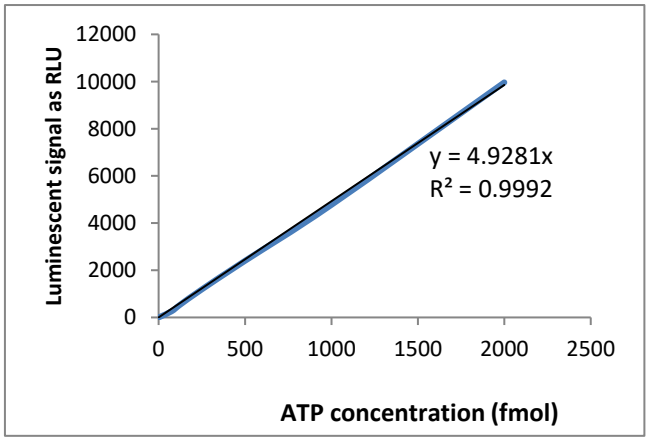

Figure 1. The straight correlation obtained between the ATP solutions (in the combination of TAE & DMSO) and relative bioluminescent units.

Determination of CFU level

Diluting BCG samples in normal saline containing 0.5% Tween-80 before plating, raised countable CFU level to 50% ($p \leq 0.01$) in comparison to using standard normal saline (data are not shown). A better correlation between viability and ATP content is expected by the more realistic CFU.

Determination of ATP content and Correlation between RLU and CFU

For the results to be accurate, the cell-free ATP was destroyed by apyrase. ATP content of the resulting extracts was determined against the standard curve. As shown in Fig.2 ATP extraction by the combination of hot TEA and DMSO (T&D) in ten replicates of a BCG sample (4×10^6cfu/mg) resulted at 11-22% higher RLU signals ($p \leq 0.05$) than hot TEA or DMSO. Reproducibility of the method as CV for hot TAE, DMSO, and T&D were 8.3, 14.4 and 3.3 respectively. As shown in Fig.3 the relationship between the different amounts of CFU and their relative RLU was linear, which indicates the RLU reading is directly proportional to the amount of ATP extracted by TD and consequently to related CFU.

A significant correlation between ATP and CFU with a low LOQ equal to10 fmol of ATP was shown by using the EnSure luminometer. The sensitivity and repeatability were evaluated by measuring each sample 10 times. These results revealed a higher and more real CFU values for the BCG samples.

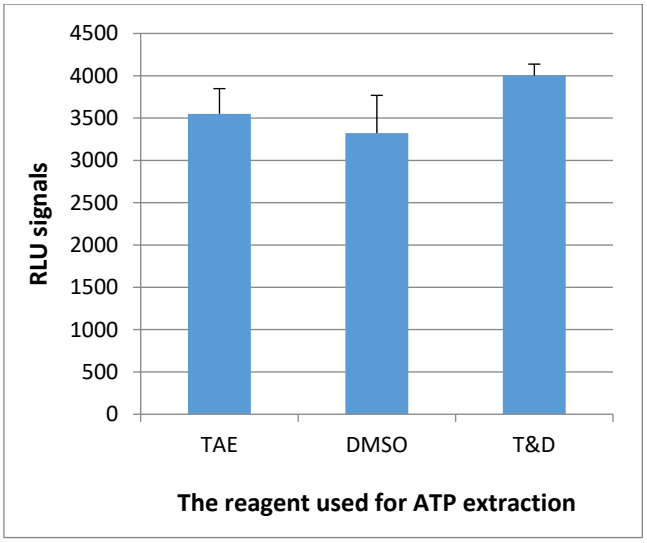

Figure 2. ATP measured as RLU signal in 10 replicates of BCG lysate in hot TAE buffer, DMSO, and combination of TAE & DMSO.

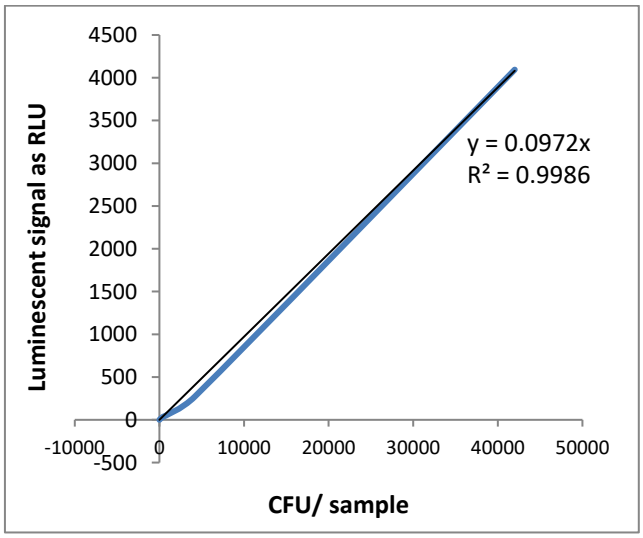

Figure 3. Regression fit of ATP bioluminescence vs CFU of diluted BCG sample. The average ratio of RLUs to ATP concentration for the EnSure system was found to be 3.5 RLU ≈ 1 fmol ATP and approxmately10 RLU equal to 1 CFU.

Discussion

The LOQ of ATP achieved by the use of the EnSure system with T&D buffer showing an approximately 100 times higher compared to the routine systems which were used in the experiments conducted by Ugarova et.al, 2016 [7] and Kolibab et.al, 2012 [1]. This indicated this system is suitable for measuring the low CFU values that are important especially in investigation studies.

Although CFU method had been the method of choice for years, since it is time-consuming and said to be variable due to tendency of BCGs to clump together; ATP assay as the main alternative rapid method has been considered to quantify the viability of BCG vaccine while it has also some variability as it was mentioned earlier. Apart from WHO endorsement of ATP assay; improvement of it assay to be more accurate, has been known necessary. In the first collaborative trial for validation of ATP assay conducted in 2008 however, only 3 out of 7 laboratories managed to obtain close results from the same samples [7].

There is a mismatch between the limit of detection (LOD) and the limit of quantification (LOQ) of the current ATP assays and the minimum level of bacteria which is needed to estimate the viability of the BCG vaccine accurately and, so there was a need for a more sensitive approach. In current ATP assays to enumerate viable BCGs [1, 6-8], the LOQ of ATP according to related standard curves (usually 10 pmol/mg) is higher than the amount of ATP exist in the prepared BCG sample even in the most concentrate one (1.8-100 µg). So a poor RLU from a highly diluted BCG sample cannot be read confidentially by the standard curves and consequently, it would not estimate the amount of live mass appropriately in a vial of vaccine usually containing $1\text{-}8\times10^6$cfu/mg. Therefore, a modified ATP extraction method and Hygiena

ATP reader system with higher sample loading capacity and more sensitivity have been used in this study to overcome the limitation.

The general method described for ATP extraction from BCG cells [5-7] including heating the samples in hot EDTA- Tris-acetate buffer. Ugarova et.al, 2016 [7] have climbed a simple and more effectual ATP extraction method by treating the sample with 80% DMSO at room temperature. Regarding the above, it is conclusive from both methods that the amount of sample and the value of ATP extracted from each one does not seem to be enough and suitable for an accurately quantifying the CFU of BCG bacilli to evaluate the vaccine for release. The LOD in each of these methods is about 10-30 pg of ATP equal to 10^5 CFU; less than the CFU in their related samples prepared for ATP extraction which is about 10^4 CFU. Considering the above and due to the low LOD of these methods, they are more suitable for measurement of CFU in reached samples such as BCG bulk than the vaccine for confirmation of Bulk before being used for vaccine production.

In this experiment, the new ATP assay by treating the cells by hot TEA & DMSO and measuring ATP by Hygiena luminometer showed to be not only more rapid and robust in repeatability and accuracy but also more effective than previous methods for the intended use. EnSure ATP assay portable luminometer (USA) has designed to keep cost low, with fewer components that mean less error from manufacturing variance, limit variability and increasing the accuracy; so resulting in repeatable ATP readings. It has evaluated for key performance metrics including linearity, repeatability, sensitivity, and accuracy by some reference laboratories as claimed by its company.

To further improve the ATP assay, simultaneously chemical and physical treatment such as using ball mill or sonication to disperse the cells completely and release more ATP can be considered as future research.

Conclusion

In addition to the importance of accurately measuring viability for the BCG vaccine; for the BCG bulk before preparation of final bulk, it could be reassuring to reach a pre-confirmed vaccine. Our method for extracting ATP and using the Portable EnSure ATP measuring system worked well and offers benefits including the fast and accurate results, thus, this method is suitable for quantifying the absolute ATP concentration inside BCG cells. Finally, whether ATP or CFU amount represents a real measurement of BCG viability cannot be determined, since the CFU method itself as the criterion to qualify the ATP method is highly variable.

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